Short Communication

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Definition of extracellular localized epitopes of Hsp70 involved in an NK immune response

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Abstract In order to define extracellular localized epitopes of Hsp70 on human tumor cells which are accessible to the immune system, six commercially available Hsp70-specific monoclonal antibodies (mAb) with different recognition sites were examined by immunological approaches. The recognition pattern of these antibodies was analyzed on purified recombinant Hsp70 proteins (rHsp70, Hsc70, DnaK), on lysates of Hsp70-expressing colon carcinoma cells (CX+) and on lysates of M21 rat-1 cells that overexpress human Hsp70 or Hsp70 fragments: ΔBgl (del 120–428) consisting of the C-terminal part and ΔSma (del 438–618) consisting of the N-terminal part of human Hsp70. All antibodies reacted equally well with rHsp70 and cytoplasmic Hsp70 derived from human tumor cells or M21 rat-1 cells. Only one antibody (MA3–007; Hsp70, Hsc70) detects a region localized within the ATPase domain of Hsp70 (amino acid 122–264) and reacts positively with the C-terminal deletion mutant ΔSma. All other antibodies, including RPN1197 are directed against the C-terminal peptide binding domain of Hsp70 and react positively with the N-terminal deletion mutant ΔBgl. Although all six antibodies detect full-length Hsp70 protein, derived from plasma membrane fractions of CX+ tumor cells, cell surface expressed Hsp70 on viable CX+ tumor cells, as determined by flowcytometry, is only recognized with the antibodies MA3–006 (Hsp70, Hsc70; 504–617), MA3–009 (Hsp70; 504–617) and RPN1197 (Hsp70). An estimation of the ratio of membrane-bound to cytoplasmic Hsp70 molecules revealed that 15–20% of total Hsp70 molecules are expressed on the plasma membrane. This tumor-selective cell surface expression of Hsp70 correlates with an increased sensitivity to lysis mediated by non-MHC restricted natural killer (NK) cells. We demonstrate that only antibodies directed against membrane-bound Hsp70 (MA3–006, MA3–009, RPN1197) inhibit NK-killing activity against Hsp70-expressing tumor cells. Taken together our data indicate that at least the C-terminal region 504–617, that contains at least one single α-helix (amino acid 512–536), has to be localized extracellularly and might be of importance for an NK-mediated anti-tumor immune response.

INTRODUCTION

Cancer immunity can be induced by intracellular localized molecular chaperones with a molecular weight of 70 kDa and of 90 kDa (DeNagel and Pierce 1993; Feldweg and Srivastava 1993; Palladino 1987; Multhoff et al 1995a; Multhoff and Hightower 1996). Besides their chaperoning function for tumor-derived peptides (Udono and Srivastava 1993; Suto and Srivastava 1995), Hsp70, the major stress-inducible member of the 70 kDa heat shock protein family (Hsp70), elicits an immune response against cancer if it is expressed on the plasma membrane and thus becomes accessible to immunocompetent effector cells (Tamura et al 1993; Yoshino et al 1994; Multhoff et al 1995a, 1997; Botzler et al 1996a; Wei et al 1996). Although the mechanism for transport and anchorage to the plasma membrane remains to be elucidated for Hsp70 that lacks a classical leader sequence, cell surface expression has been demonstrated on various human tumor cell types by flowcytometry, by light and electronmicroscopy (EM), and/or by selective cell surface protein biotinylation

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either under physiological conditions or following heat/chemical stress (Ferrarini et al 1992; Tsuboi et al 1994; Multhoff 1995a; Botzler et al 1996a, 1996b; Multhoff et al 1997). Normal cells including PBL, fibroblasts or endothelial cells derived from healthy human individuals, fail to express Hsp70 on their plasma membrane (Multhoff et al 1995b).

It has been reported that NK cells are important players in the recognition and killing of cancer cells (Pross et al 1993). We have demonstrated that cell surface expression of Hsp70 on certain tumor cells, correlates with an increased sensitivity to lysis mediated by non-MHC-restricted NK cells (Multhoff et al 1995a, 1997; Botzler et al 1996a). Antibody blocking studies using Hsp70-specific monoclonal antibodies revealed a strong inhibition of this NK-mediated tumor cell lysis (Multhoff et al 1997). In the present study we were interested to know which part of human Hsp70 is localized extracellularly and thus might be involved in the induction of an NK-mediated immune response. For this purpose we tested six commercially available antibodies directed against different epitopes of Hsp70 by immunological approaches. Using electronmicroscopy an estimation of the ratio of cell membrane bound to cytoplasmic Hsp70 molecules has been determined.

RESULTS AND DISCUSSION

Definition of extracellular localized immunogenic epitopes of Hsp70

Immunoblots were performed using

1. purified human recombinant Hsp70 protein (rHsp70), Hsc70 and DnaK the *Escherichia coli* analogue of human Hsp70
2. lysates of M21 rat-1 cells expressing deletion mutants of human Hsp70 that either lack or express the N- or C-terminal part (kindly provided by Dr Gloria Li; Li et al 1992, 1995)
3. tumor cell lysates derived from CX+ colon carcinoma cells that have been shown to express Hsp70 on their plasma membrane (Multhoff et al 1997).

A description of the antibodies including their names, binding sites as determined by commercial claims, species specificities and isotypes, that were used for immunoblotting, flowcytometry and antibody inhibition assays is shown in Table 1. Our data summarized in Table 2 and Table 3 indicate that all tested antibodies react equally well with recombinant Hsp70 protein (SPP-755, kindly provided by Dr Lee Mizzen, StressGen) and with Hsp70 derived from lysates of CX+ tumor cells or of M21 rat-1 cells that are constitutively overexpressing human Hsp70 (Li et al 1995). A specific recognition of the stress-inducible

Table 1 Description of mAb directed against different epitopes of Hsp70 as determined by commercial claims

Antibody name	Binding site	Species reactivity	Isotype	Clone
MA3–007	122–264	Human, rodent	IgG	5A5
SPA-810	437–504	Human, rodent	IgG1	C92F3A-5
MA3–008	437–479	Human, rodent	IgM	2A4
MA3–006	504–617	Human, rodent	IgG1	3A3
MA3–009	504–617	Human	IgG	4G4
RPN1197	?	Human, rodent	IgG1	Ag4–1

Antibodies with specificity for Hsp70 that were used for immunoblotting and phenotypic characterization: RPN1197 (Amersham; Welch and Fermascio 1985), MA3–007 (ABR; Sarge et al 1993), MA3–006 (mAb, kindly provided by Dr S. Fox, Northwestern University, Chicago IL, USA), MA3–008 (ABR; Murphy et al, unpublished observation), MA3–009 (ABR; Murphy et al, unpublished observations), SPA810 (StressGen; Welch and Suhan 1986). The boundaries of the antibody epitopes are derived from commercial claims (ABR, StressGen). The epitope of the antibody RPN1197 (Amersham) has not been defined yet.

Table 2 Binding of different Hsp70-specific mAb to recombinant Hsp70, Hsc70 or DnaK the Hsp70 analogue in *E. coli* (Schlesinger et al 1982; Feige and Polla 1994) as determined by dot blot analysis

Antibody	Binding site	rHsp70	Hsc70	DnaK
MA3–007	122–264	+	+	–
SPA-810	437–504	+	–	–
MA3–008	437–479	+	+	–
MA3–006	504–617	+	+	–
MA3–009	504–617	+	–	–
RPN1197	?	+	–	–

Following transfer of purified Hsp70 proteins (rHsp70, Hsc70, DnaK) at a concentration of 40, 20 and 10 ng onto NC membranes, and blocking of non-specific binding sites (5% skim-milk PBS), the following Hsp70-specific mAb, diluted 1:2000 in PBS/0.5%Tween/1%skim-milk, were used for immunoblotting: MA3–006 (mAb, kindly provided by Dr S. Fox, Northwestern University, Chicago IL, USA), SPA810, MA3–008, MA3–006, MA3–009, RPN1197. IgG/M isotype-matched antibodies, used as controls (Dianova, Hamburg, Germany) did not show any positive reactivity with either one of the proteins.

+: indicates a positive reactivity of the antibodies with the Hsp70 protein at each protein concentration ranging from 40 ng down to 10 ng.

Hsp70 without cross-reactivity to Hsc70 (SPP-750, kindly provided by Dr Lee Mizzen, StressGen) was observed with the antibodies SPA810, MA3–009 and RPN1197 (Table 2). All other antibodies (MA3–007, MA3–008, MA3–006) revealed a strong cross-reactivity with Hsc70 purified from bovine brain. None of the tested antibodies reacted positively with DnaK (SPP-630–2, kindly provided by Dr Lee Mizzen, StressGen) the Hsp70 analogue derived from *E. coli* that exhibits only minor homology to Hsp70 (Schlesinger et al 1982; Table 2).

As shown in Table 3, MA3–007 (Hsp70, Hsc70; 122–264) is the only antibody that reacts with an epitope localized in the highly conserved ATPase domain of Hsp70. The 44kDa ATPase region (1–386) of Hsp70 and Hsc70, as determined by X-ray crystallography, consists

Table 3 Binding of different Hsp70-specific mAb to lysates of tumor cells (CX+) and of M21 rat-1 cells overexpressing either human Hsp70, or an Hsp70 fragment lacking the N-terminal ATPase domain (deletion mutant ΔBgl) or the C-terminal substrate binding domain (deletion mutant ΔSma) as determined by Western blot analysis

Antibody	Binding site	CX+ Hsp70 intact	M21 Hsp70 intact	ΔBgl Hsp70 del 120–428	ΔSma Hsp70 del 438–618
MA3–007	122–264	+	+	–	+
SPA-810	437–504	+	+	+	–
MA3–008	437–479	+	+	+	–
MA3–006	504–617	+	+	+	–
MA3–009	504–617	+	+	+	–
RPN1197	428–640	+	+	+	–

The Hsp70-specific mAb diluted 1:2000 in PBS/0.5% Tween/1% skim-milk were used for Western blot analysis of cell lysates generated from CX+ tumor cells, M21 rat-1 cells that are constitutively overexpressing human Hsp70, or Hsp70 fragments that either lack the N-terminal region 120–428 (ΔBgl) or the C-terminal region 438–618 (ΔSma). The deletion mutants for human Hsp70 have been kindly provided by G. Li (Li et al 1995). Briefly, exponentially growing cells (5×10^6) were harvested and lysed in 0.5% NP40 solution. Equal protein amounts (10 µg) were run on a 10% SDS-PAGE under reducing conditions and transferred to Immobilon PVDF membranes. Following incubation with the primary Hsp70-specific mAb, immune complexes were detected using the ECL system (Amersham).
+: indicates a positive signal in Western blot analysis.

of four domains forming two lobes with a cleft for bonding of nucleotides (McKay et al 1994; Morshauser et al 1995). Upon ATP binding a conformational change is detectable (Wilbanks et al 1995) that affects the affinity of binding substrates (Palleros et al 1993). Our data show a positive reactivity of MA3–007 with the Hsp70 deletion mutant ΔSma that lacks the C-terminal part 438–618. Since only one out of six Hsp70-specific antibodies is directed against the N-terminal part, we speculate that the highly conserved ATPase domain is less immunogenic compared to the substrate binding domain. MA3–008 (Hsp70, Hsc70; 437–479) that exhibits overlapping regions with SPA810 (Hsp70; 437–504) and the antibodies MA3–006 (Hsp70, Hsc70; 504–617), MA3–009 (Hsp70, Hsc70; 504–617) all react with the C-terminal part of Hsp70. With the exception of MA3–007, all antibodies, including RPN1197, recognize the deletion mutant ΔBgl that contains the C-terminal region 428–617 but lacks the N-terminal part 120–428, as shown by Western blot analysis of cell lysates (Table 3). With respect to these findings, we conclude that the yet undefined recognition site of RPN1197 has to be localized within the C-terminal part 428–640.

Definition of extracellular localized epitopes of Hsp70 on tumor cells that are involved in the recognition by NK cells

In order to define extracellular localized Hsp70 epitopes that are accessible to cells of the immune system all antibodies were tested by flowcytometry on viable CX+ and as a control on CX– tumor cells, that have been shown to express Hsp70 only on about 20% of the cells (Multhoff et al 1997). Despite the fact that all antibodies reacted positively with cytoplasmic Hsp70 generated from cell lysates (CX+, M21 rat-1 cells; Table 3), plasma membrane expression of Hsp70 was detectable only with the antibodies MA3–006 (Hsp70, Hsc70; 504–617), MA3–009 (Hsp70; 504–617) and RPN1197 (Hsp70; C-terminal part 428–640). The antibody with a recognition site within the ATPase domain (amino acid 122–264), was unable to recognize membrane-bound Hsp70 on viable CX+ tumor cells. Furthermore, antibodies (SPA-810; MA3–008) directed against the peptide binding region 437–503 also failed to detect cell surface-expressed Hsp70 on viable tumor cells. The binding pattern of these antibodies was comparable to that of the isotype-matched control antibody (Table 3). Regarding these findings we speculated that only the C-terminal part 504–617 of the Hsp70 protein is expressed on the plasma membrane. However, this hypothesis could be excluded since the complete protein could be immunoprecipitated from the plasma membrane following selective cell surface biotinylation of viable CX+ tumor cells (Multhoff et al 1997) and since the protein bands immunoprecipitated from the membrane fraction of Hsp70-expressing tumor cells revealed a molecular mass of 72kDa (Multhoff et al 1995b).

In order to get an estimation about the ratio of the amount of membrane-expressed to cytoplasmic Hsp70 proteins, the number of gold particles was counted on 10 independent electronmicroscopical sections that had been stained with Hsp70-specific mAb RPN1197 (Amersham) under saturating conditions (mAb dilution 1:200/grid containing 5–10 cell sections). One representative cryo-electronmicrograph at different magnifications (A: overview × 6400 and B: section × 19 000) is shown in Figure 1. The calculated data indicate that between 15 and 20% of total Hsp70 molecules are expressed on the plasma membrane of CX+ cells.

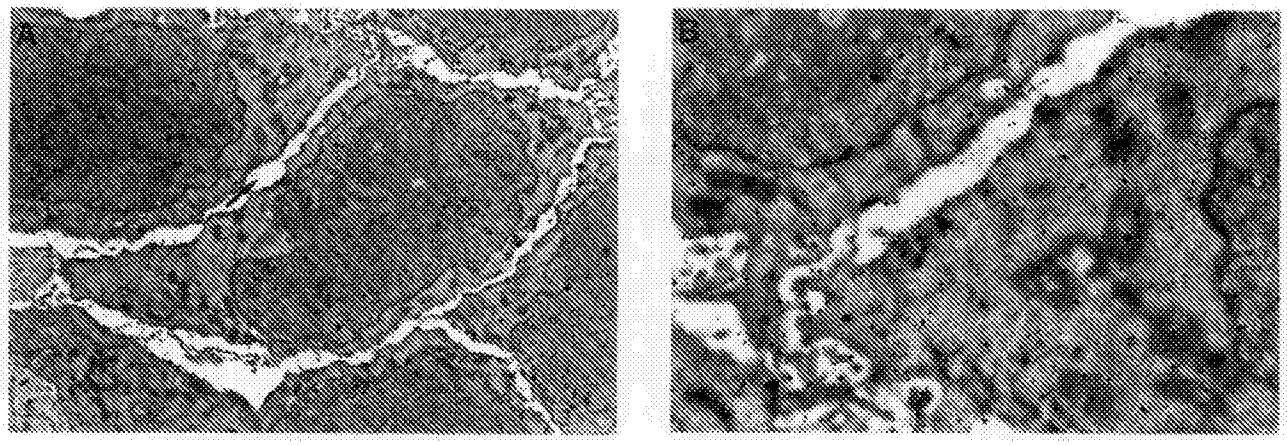

Fig. 1 Cryo-ultramicrotomy image of CX+ tumor cells labeled with Hsp70 antibody at saturating conditions. The frozen sections were exposed to Hsp70-specific mAb (RPN1197) followed by protein A-gold labeling (10 nm, Aurion) and viewed in a Zeiss EM 10CR electron microscope. With this approach intra- and extracellular localized staining of antiges is possible. The percentage of cell surface-expressed Hsp70 was determined by counting and calculating of gold particles on the plasma membrane and inside the cell on 10 randomly selected sections of different CX+ tumor cells. The ratio of membrane-bound Hsp70 was calculated according to the following formula: % cell membrane-bound Hsp70 molecules = [number of Hsp70 gold particles on the plasma membrane/total number of gold particles] × 100.

The values were: 74/417: 17.7%; 10/68: 14.7%; 55/275: 20.0%; 25/175: 14.3%; 38/238: 15.9%; 36/225: 16.0%; 41/249: 16.5%; 81/442: 18.3%; 25/140: 17.8%, 25/165: 15.1%.

One representative electronmicrograph is shown, (A) denotes an overview of complete CX+ cells (× 6400); (B) denotes two sections out of these cells (× 19 000). An arrow marks the section area that was chosen.

Table 4 Binding of different Hsp70-specific mAb to viable Hsp70-expressing (CX+) and non-expressing (CX–) tumor cells and inhibition of lysis of CX+ cells by these mAb

Antibody	Binding site	Membrane Hsp 70 on CX+ cells	Membrane Hsp70 on CX– cells	Rate of Inhibition Mean values	± SD
IgG/M	neg. ctrl.	4%	3%	1.21	±0.13
MA3–007	122–264	5%	2%	1.08	±0.20
SPA-810	437–503	3%	3%	1.16	±0.16
MA3–008	437–479	2%	4%	1.21	±0.41
MA3–006	504–617	92%	25%	1.71*	±0.40
MA3–009	504–617	87%	24%	1.60*	±0.22
RPN1197	428–640	91%	20%	2.21*	±0.31

The human autologous colon carcinoma sublines CX+ and CX– (Multhoff et al 1997) were used for flowcytometry analysis using different Hsp70-specific mAb at a final concentration of 1 µg/1 × 10^6 cells. The flowcytometry data are expressed as the percentage of positively stained cells as determined on a FACScan instrument (Becton Dickinson, Heidelberg, Germany) minus the percentage of cells determined with an isotype-matched control antibody (Dianova, Hamburg, Germany).

A comparison of the sensitivity to lysis of CX+ tumor cells either untreated or following pre-incubation with HSP70 antibodies at a final concentration of 5 µg/1 × 10^6 cells (saturated conditions). Inhibition assays were performed using NK-enriched effector cells (CD3:11%; CD16/CD56:54%) at effector to target ratios ranging from 40:1 to 5:1 as determined in a 4 h ^{51}Cr radioisotope assay (Multhoff et al 1995a). NK cells were prepared according to a modified method of Vujanovic (1995), as described in the results. The data represent the mean values of the calculation of the relative rate of inhibition of CX+ cells either untreated or treated with mAb at saturating conditions ± SD.

*: indicates a significant inhibition of lysis at all titration steps compared to control antibody blocking ($P < 0.05$).

An involvement of Hsp70 in the NK-mediated immune response against cancer cells has been shown based upon its expression on the surface of these cells (Multhoff et al 1995a). The antibody RPN1197 directed against plasma membrane-bound Hsp70 efficiently inhibits the lysis of Hsp70-expressing tumor cells (Botzler et al 1996a; Multhoff et al 1997), whereas antibodies directed against MHC class I and II or adhesion molecules (ICAM, NCAM) exhibited no inhibitory effect on tumor cell lysis. Here, we demonstrate by a comparative analysis of Hsp70-specific antibodies with different binding epitopes that only antibodies directed against the C-terminal region 504–617, efficiently inhibit the lysis of Hsp70-expressing CX+ tumor cells that was mediated by NK cells. This region is predicted to contain α-helices (Boice and Hightower 1997) and one helix was confirmed (Morshauser et al 1995). No significant inhibition of lysis was observed by antibody blocking studies using Hsp70-specific antibodies on CX– tumor cells (data not shown). For antibody blocking studies, NK cells were generated from monocyte-depleted PBMC, following stimulation with IL-2 (100 IU/ml kindly provided by Chiron). Briefly, after separation of PBL into an adherent NK population and a non-adherent T cell fraction, (Vujanovic et al 1995)

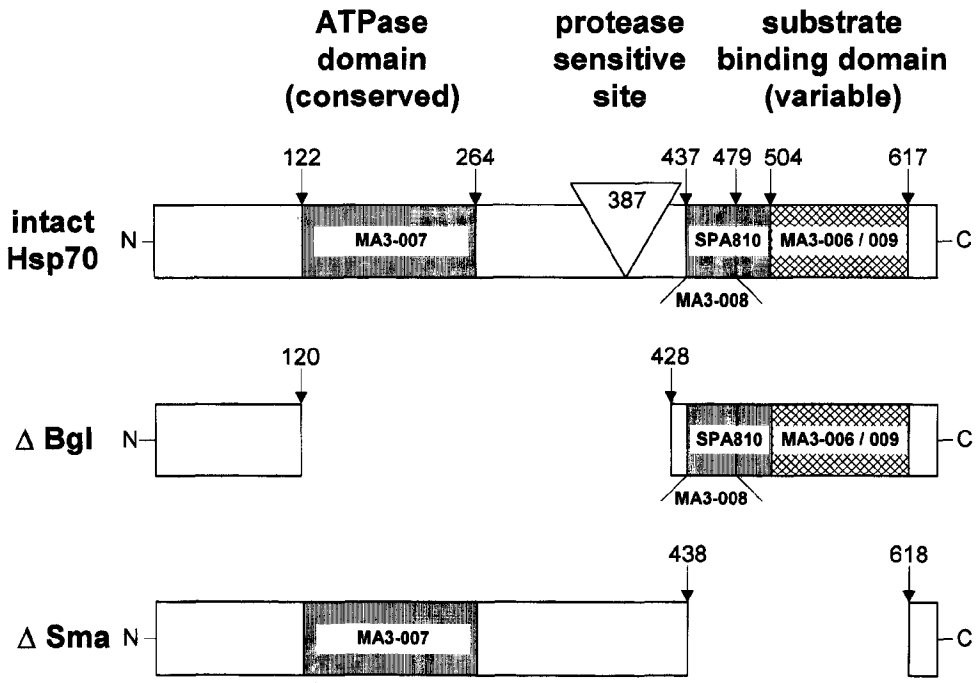

Fig. 2 Schematic illustration of antibody binding sites of different Hsp70-specific mAb (MA3–007, MA3–008, SPA810, MA3–006 and MA3–009) on the primary amino acid sequence of intact Hsp70 and on the deletion mutants ΔBgl, that lacks the N-terminal region 120–428, and ΔSma that lacks the C-terminal region 438–618. Antibody recognition sites are marked as dotted and hatched areas. Dotted regions indicate either intracellular localized, or extracellular hidden antibody epitopes; hatched regions indicate extracellular accessible regions of Hsp70 that are involved in NK killing of Hsp70-expressing tumor cells as determined by FACScan analysis and by antibody blocking cytotoxicity assays.

a further purification of the NK cell population was obtained by a sequential depletion of CD3+ T cells and CD14+ monocytes (CD3, CD14 antibodies; Dianova, Hamburg, Germany) using the magnetic bead separation method. The purity of the NK population was determined by flowcytometry using antibodies directed against CD3/CD16/CD56 (mAb, Becton Dickinson, Heidelberg, Germany). Antibodies recognizing the highly conserved N-terminal ATPase region or the peptide-binding domain 437–503 of Hsp70 (McKay et al 1994; Morshauser et al 1995) did not affect NK killing activity (Table 4). As a control, the lysis of tumor cells that lack Hsp70 on their plasma membrane was not affected by these antibodies (Multhoff et al 1995a, 1997). A schematic illustration of the epitope mapping analysis of all tested Hsp70-specific antibodies on the primary amino acid sequence of Hsp70 and on the deletion mutants ΔBgl and ΔSma is summarized in Figure 2. Antibody recognition sites marked as hatched areas indicate extracellular localized epitopes of Hsp70 that might be involved in the recognition of Hsp70-expressing tumor cells by NK cells. In summary, our data indicate that C-terminal antibody recognition epitope 504–617 containing at least one α-helix is also important for NK recognition.

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